Stress Induced Hyperthermia in zebrafish: a reply to Key et al.

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Key et al. make several criticisms of the paper "Fish can show emotional fever: stress-induced hyperthermia in zebrafish" by Rey et al. 2015 [1]. The authors reply to these (italicised) more-or-less in their order in Key et al., before making some general comments.

Rey et al. state that "...lack of emotional fever in fishes..." would reflect "...a lack of consciousness.... The cited statement refers to the views of Cabanac [4] and others and in the original is preceded by the phrase "According to this view ...", making it clear that the authors were simply reporting, not supporting, the view. By omitting this qualification, Key et al. ascribe to Rey et al. [1] an opinion about the relationship between SIH and consciousness that they did not express.

Rey et al. are clearly inferring that their results are consistent with consciousness in fishes. Rey et al [1] were careful not to suggest this, merely stating that the demonstration of SIH in zebrafish removed one line of argument for lack of consciousness in this group.

Both these points are discussed in more detail below.

Key et al. make a number of criticisms of how space use was quantified and analysed;

Data were collected during brief periods that amount to only 1.67% of the total observation time: Data were extracted from the videos by scan sampling, a well-validated method for quantifying behaviour over extended periods [6].

… the data cannot distinguish between the possibilities that the same fish entered and remained in the hyperthermic chambers versus... all experimental fish (or a subset..) moved into and out of the hyperthermic chambers. This is correct. Rey et al. [1] explicitly discuss the fact that their results refer to groups and not individuals and advocate further studies at the individual level. It is relevant that the videos showed fish in all groups moving frequently between chambers in both directions, making some use of most of the tank, so there was a turnover of fish in all chambers.

Our analysis suggests that there were only ~2 more fish in hyperthermic chambers 5 and 6 compared to controls at any particular moment …. These .. changes …are modest, (and) not statistically significant. This is based on a reconstruction of fish distributions from summary data in Supplementary Figure 2. In the authors' view, this is inappropriate, much of the information contained in the raw data being lost, giving the analysis poor power for discriminating treatment difference. While wrong in suggesting that the difference between control and confined groups were not significant (see below), Key et al. are right in that actual data showed only a minority of fish in two warmest chambers at any one time, median numbers being 2 and 4.5 in control and confined groups respectively. In addition to the overall movement of the groups evident from the videos, this represents more than a doubling of the number of fish in the 2 warmest compartments. Equivalent figures for the top three warmer compartments are 6 for control and 9 for confined groups, a 50% increase in use of the warmer chambers.

In their concluding paragraph, Key et al. refer to *".. the weak and possibly inappropriate statistical analyses (in particular, … pooling of dependent samples over time and … analysis by a Mann-Whitney-U test for independent samples…)"*

Rey et al. [1] carried out an initial highly conservative analysis, accommodating non-independence of fish within groups and across samples by using a single measure of space for each group (median proportion of fish in the 3 warmer chambers across all samples). This measure was compared using a Mann-Whitney test for independent samples. The main analysis used a multilevel Poisson regression, a powerful approach tailored to the analysis of count data that deals with non-independence by treating groups as the statistical unit and counts within chambers as a repeated measurement on the statistical unit, with further repeated measurements across time. Both analyses were appropriate and both showed a significant treatment effect.

Key et al. raise several points relating to possible alternative explanations for the different fish distributions:

"In particular, the fish may have been responding to various substances secreted by their companions (e.g alarm substance and/or water borne cortisol)." The authors recognise that stress responses are transmitted within shoals [7], sometimes via water-borne chemicals [8]. However, the aim was to compare unstressed fish with definitely stressed ones, so if the response to confinement was amplified in this way, this would not negate the original conclusion.

"Immediate erratic/escape responses elicited by such substances and/or subsequent avoidance of the compartment in which stress was imposed could explain the observed change in distribution." Short-term stress-related responses were certainly observed, but only immediately after release from the net. To avoid these distorting the results, the first sample after confinement was omitted from the analyses.

The suggestion that the fish were avoiding the chamber in which they were confined (Chamber 3) is interesting and would be plausible had confined groups made greater use of the compartments in both directions. However, this was not the case, post-confinement distributions in all 3 groups being centred on the warmer chambers. In addition, the videos showed fish in the confined groups making frequent voluntary movements into chamber 3, which is not consistent with avoidance of this chamber. Rey et al. [1] did not include this information in their original paper and welcome this opportunity to present it here.

Key et al. suggest an explanation as to why fish might have moved to warmer chambers, thus: *"The reported small distribution shift suggests fish moved towards their preferred normal rearing temperature in chamber 4 and occasionally explored chambers 5 and 6 while avoiding chamber 3."*

This assumes that zebrafish prefer the temperature at which they have been reared, but the literature provides no support for this assumption. In fish generally, temperature preferences are influenced by a variety of factors (e.g. current growth rates/metabolic scope [9] and time since feeding [10]), not all of which are amenable to control. This being the case, the best way to predict preferred temperature for any given fish in any given experiment is to use the demonstrated preference of fish from the same source in the same set-up, reared identically up to the point of intervention. In the temperature gradient used by Rey et al. [1], unstressed groups preferred chamber 3, so the experimental groups were confined at the preferred temperature for zebrafish from this source in these conditions. The suggestion that the confined fish moved towards their preferred water temperature, rather than specifically moving into warmer water, therefore does not hold up.

FOLLOWING AN EMAIL EXCHANGE BETWEEN FELICITY AND SONIA ABOUT WHAT THE LITERATURE ACTUALLY SAYS, I SENT AN EMAIL WITH THE FOLLOWING SUGGESTED REPLACEMENT PARA – as ever, edited a bit from my original, AND AN EDITED VERSION OF THE LETTER TO ACCOMODATE THIS.

The authors refute Key et al.'s assumption that the preferred temperature of the zebrafish in their study would be their rearing temperature. After overnight acclimation in the temperature gradient, chamber use would reflect (be approaching??) the final preferendum [X] and for unstressed fish this final preferendum in Rey et al.'s experimental set-up [1] was the temperature of chamber 3 (26.92oC). The experimental fish were of identical provenance and reared identically up to the point of confinement, so there is no reason to expect their preference to be different from controls. They were therefore confined at their current preferred temperature, so in our view the suggestion that they moved towards their preferred water temperature, rather than specifically moving into warmer water, does not hold up.

"Rey et al. [1] … provide no evidence that the purported altered thermal preference by net-confined zebrafish is driven by fish experiencing conscious anxious states….". Rey et al. [1] make no statements or assumptions about whether the confined fish were experiencing anxiety, conscious or otherwise, merely that they were stressed.

In their concluding paragraph Key et al. state that Rey et al. [1] provide an *incomplete description of methodology*. Nowhere in their critique do they refer to specific points where more information is needed, making it impossible for the authors to accept or refute the criticism.

On re-reading the target paper [1] with Key et al.'s comments in mind, there are some points that perhaps could have been made more clearly and the authors welcome the opportunity to clarify these. The still-influential statement that fish, as a group, do not show emotional fever arises from Canabac and Laberge [11] finding that goldfish fail to adjust their temperature preference upwards when stressed. Rey et al. [1] showed that, when allowed to express natural, fine-scale preferences in a temperature gradient, zebrafish do exactly this. The logical link made by Cabanac *et al* between SIH/emotional fever and consciousness is obscure [4], so of course showing SIH in zebrafish does not prove the existence of conscious states in fish. Whether and/or to what extent fish are conscious is a complex and difficult question, the answer to which will come from detailed and careful research from both a neurobiological [e.g. 12] and a behavioural/psychological [e.g. 13] perspective and will almost certainly vary among fish species. Rey et al.'s demonstration of SIH in zebrafish does not contribute to this research effort directly, but it does contribute indirectly to the broader debate by removing one particular piece of evidence for lack of consciousness in fish.

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